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PRINCIPAL INVESTIGATOR: Young C. Lin, Ph.D.

CONTRACTING ORGANIZATION: Ohio State University
Columbus, Ohio 43210-1092

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Young C. Lin Ph.D.

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Ohio State University
Columbus, Ohio 43210-1092

E-Mail: lin.15@osu.edu

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Background:

The use of anabolic agents has become an important practice in the meat-producing industry, as such agents increase weight gain by enhancing protein deposition and improving feed conversion, as well as increasing the muscle-to-fat ratio. As a result, feed costs are reduced and the quality of meat is improved. These anabolic agents have biological activities in common with natural estrogenic, androgenic and progestogenic steroid hormones, which have potential effects on protein metabolism. The presence of trace amounts of natural steroid hormones in foodstuffs consumed by humans are easily degraded by the liver, therefore the concern that these natural compounds may pose a health risk to humans is considered negligible.

Our research project has focused on the synthetic anabolic compound, zeranol (Ralgro®), which is a non-steroidal agent possessing estrogenic action similar to the natural estrogens. Zeranol is approved by the Food and Drug Administration (FDA) as a growth promoter for use in the beef, veal, and lamb industries in the U.S. Administration of zeranol is by the subcutaneous implantation in the ear of a pellet containing 36 mg of zeranol per beef heifer followed by a second pellet 30 days later. Thus, people are exposed to Z as a consequence of the direct and intentional introduction of the compound into food animals by veterinary professionals on behalf of beef industry farmers. The motivation for our research is our concern is that the consumption of food products, particularly beef, derived from food animals treated with Z has a potential health impact on human consumers, particularly with respect to reproductive endocrinology and hormone-sensitive organs. Our experimental data generated from research supported by the U.S. Army Medical Research and Materiel Command (USAMRMC) Breast Cancer Research Program supports this concern by providing a putative link between Z and risk for human breast cancer growth. The findings of our funded research are described below.

Zeranol and human health risk:**A. Evidence from cell culture studies:*****1. Mitogenic activity is present within biological specimens derived from zeranol-implanted beef cattle:***

Beef cattle were implanted with two zeranol pellets (36 mg zeranol/pellet; sc in the ear) 30 days apart. Serum, muscle (meat) and adipose samples were collected 60 days after implantation of the first pellet. Extracts from the equivalent of 44.4 mg of meat and adipose tissue were prepared. Incorporation of ³H-thymidine was measured to determine the effects of these biological samples on DNA synthesis in various human breast and breast cancer cell types.

Results from primary cultured normal human breast cells. After 24 hours of treatment with 0, 1, 5, and 25% (v/v) zeranol-containing serum, DNA synthesis in primary cultured normal human breast cells was significantly elevated in a dose-related fashion (25, 70, 180 and 370 DPM/μg cell protein/6 hours, respectively). Similarly, 24 hours of treatment with the meat and adipose extracts (derived from 44.4 mg of tissue) induced significant elevations in DNA synthesis (27 and 67% increases, respectively). Furthermore, when the primary cultured normal human breast cells were treated with a range of dose levels of the meat extract (corresponding to 0, 0.34, 1.70 and 8.5 ng of zeranol/ml, as determined by HPLC), DNA synthesis was elevated in a dose-dependent manner (0.05, 1, 4.1 and 10.3 DPM/well (10³), respectively).

Results from human breast cancer cell lines. The estrogen receptor (ER)-positive and -negative cell lines MCF-7 and MDA-MB-231, respectively, were utilized to determine whether the effect of zeranol-containing biological samples on DNA synthesis is dependent on ER status. Treatment with 1 and 5% zeranol-containing serum resulted in statistically significant elevations in DNA synthesis (1500 and 1800 DPM/μg protein/6 hours, respectively) as compared to control (500 DPM/μg protein/6 hours) in MCF-7 cells. The same treatments did not change levels of DNA synthesis in MDA-MB-231 cells.

Significance. These data imply that serum and tissue levels of zeranol or zeranol residues/metabolites are

capable of enhancing DNA synthesis in cultured normal human breast cells, as well as human breast cancer cells. Also, meat extracts containing as little as 0.34 ng zeranol/ml are capable of stimulating DNA synthesis. These data suggest the potential for biological effects, specifically at the level of the breast, from the consumption of food products from animals treated with zeranol. It is unclear however whether the observed growth-promoting activity results from the direct action of biologically active zeranol residues on breast cells or from the ability of zeranol itself or its metabolites to modulate other not-yet-defined factor(s) within the zeranol-treated cattle which in turn are responsible for the stimulation of breast cell growth.

Of interest is the data from the meat and adipose extracts. Although these extracts were derived from equal amounts of tissue (44.4 mg), adipose tissue extract displayed a greater potency than meat extract in stimulating the increase in DNA synthesis. Since the level of HPLC-detectable zeranol that we detected in the adipose tissue of zeranol-treated cattle is lower than that in meat (3.0 vs 5.5, respectively), it is reasonable to assume that this adipose tissue contains zeranol residues/metabolites or other not-yet-defined factor(s) or substance(s) that are more biologically active than those found in meat. This is an important topic deserving of further investigation in the future, as it suggests adipose tissue of zeranol-treated cattle is capable of accumulating potent proliferative metabolite(s) or substance(s) through yet unidentified metabolic pathways. In addition, the identification of this as-yet-undefined mitogenic compound(s) would be useful in fully understanding the action of zeranol residue/metabolite-containing food items on estrogen-sensitive tissues.

The data generated from the ER-positive and -negative breast cancer cell lines indicate that the stimulatory action of zeranol-containing biological specimens on DNA synthesis is mediated by an ER-dependent pathway. The observation of a proliferative effect of zeranol-containing serum on the ER-positive MCF-7 cells, but not on the ER-negative MDA-MB-231 cells, supports this conclusion. Furthermore, the data show that primary normal human breast cells and the MCF-7 cells possess different sensitivities to the proliferative effects of zeranol-containing serum. In primary normal human breast cells, significant stimulation of DNA synthesis required treatment with 5 - 25% zeranol-containing serum, while for MCF-7 cells, 1% zeranol-containing serum was sufficient to induce proliferation. These data provide some intriguing insight into the differential action of a nonsteroidal, estrogenic compound on different human breast cell types. It remains to be determined whether such differential action also occurs under *in vivo* conditions, which is an interesting avenue for further investigation.

2. Zeranol possesses estrogenic bioactivity comparable to that of known estrogens:

Cultured human breast cells and breast cancer cells were used in a series of experiments to characterize its bioactivity in comparison to known estrogens (estradiol, diethylstilbestrol), particularly with respect to effects on gene expression. A major emphasis of this portion of our research has focused on a candidate tumor suppressor gene, protein tyrosine phosphatase-gamma (PTP γ). Specifically, we have explored the involvement of PTP γ in mammary tumorigenesis and the ability of estradiol-17 β (E2) and zeranol to regulate its expression in breast tissues and cells.

The expression of PTP γ has been shown to be reduced in human ovarian and lung tumors (van Niekerk and Poels, 1999) and in diethylstilbestrol (DES)-induced kidney tumors in Syrian hamsters (Lin et al., 1994). Recently, our laboratory demonstrated the expression of PTP γ mRNA in human breast cells, tissues and breast cancer cell lines and the down-regulation of PTP γ mRNA expression by E2 via an estrogen receptor-mediated mechanism (Zheng et al., 2000). These findings are the basis of our contention that suppression of PTP γ mRNA levels by natural estrogens or estrogenically active agents (xenoestrogens/endocrine disruptors) like zeranol, plays a role in mammary tumorigenesis. Down-regulation of PTP γ by estrogenically active compounds may be intimately related to the transformation of normal breast cells to preneoplastic or neoplastic cells and may serve as a molecular biomarker for breast cancer. This portion of the report describes our recent findings on the regulation of PTP γ expression in

human breast cells and tissues by E2 and zeranol, and the importance of epithelial-stromal interactions in this regulation. These experiments utilized both normal and cancerous human breast tissue specimens obtained from human patients through the Tissue Procurement Program of the NCI-funded Cooperative Human Tissue Network at The Ohio State University Hospital and Comprehensive Cancer Center. Primary cultured breast epithelial and stromal cells were used to determine the effects of treatment with E2 and zeranol on PTP γ expression levels. A dual chamber co-culture system was used to investigate the role of epithelial-stromal cell interactions on PTP γ expression. Expression of PTP γ mRNA and protein were evaluated by semiquantitative RT-PCR.

Results from primary cultured normal human breast epithelial cells. Epithelial cells isolated and cultured from noncancerous breast tissues from reduction mastectomy patients were treated for 24 hours with 30 nM E2 or zeranol. Both E2 and zeranol reduced PTP γ mRNA levels in human breast epithelial cells by approximately 30%. This reduction, however, was not as large as those observed in cultured human breast tissue fragments treated with E2 or zeranol (described later in this report). This finding led us to explore the role of mammary epithelial-stromal interactions in the estrogenic regulation of PTP γ .

Results from co-cultured normal human breast epithelial cells and stromal cells. Epithelial and stromal cells isolated from noncancerous breast tissues from reduction mastectomy patients were treated for 24 hours with 30 nM E2 or Z during culture in a dual chamber co-culture system. First, control epithelial cells contained greater levels of PTP γ mRNA than the corresponding stromal cells, which suggests that PTP γ is predominantly localized to the epithelium. Also, in both cell types, Z induced reductions in PTP γ mRNA expression that were similar in magnitude to those induced by E2. Finally, epithelial cells exhibited a drastically greater reduction in PTP γ mRNA expression level in response to E2 or Z than the stromal cells (80-90% vs 20-30%, respectively).

Significance of results from primary cultured cells. First, the results show that zeranol induced a suppressive response in PTP γ mRNA expression that was equivalent to that induced by E2. Thus, zeranol may induce effects in the human breast that are nearly identical to those resulting from E2 exposure. Of particular significance is that the degree of E2- or Z-induced suppression of PTP γ mRNA expression in co-cultured epithelial cells (80-90%) is much greater than the level of suppression induced in epithelial cells cultured alone (30%). Thus the result indicates that epithelial-stromal cell interaction(s) are important in the response of breast cells to estrogenically active agents, including Z, specifically as it relates to the expression of PTP γ .

Results from human breast cancer cell line, MCF-7. The ER-positive MCF-7 human breast cancer cell line was treated for 24 hours with zeranol, DES or E2 (all at 20 nM). Levels of cathepsin D mRNA were determined by RT-PCR. Zeranol, DES and E2 caused 2.1, 3.3, and 4.2-fold elevations in cathepsin D mRNA levels in MCF-7 cells.

Significance of results from MCF-7 cells. Cathepsin D is an estrogen-inducible gene which has been linked to the regulation of normal and cancerous breast cell growth. This result shows that zeranol is capable of altering the expression of an established estrogen-regulated gene in human breast cancer cells, in addition to the effects on PTP γ expression in normal human breast cells. Furthermore, the induction of gene expression helps to rule out a nonspecific cytotoxic effect of zeranol on breast cells which could have resulted in the suppression of PTP γ mRNA. Of course, since the zeranol used in this experiment was in the pure chemical form, it is difficult to compare these results with those generated from the use of meat extracts containing HPLC-detected zeranol. For example, as described above, HPLC-detected zeranol at a dose of 0.34 ng/ml is capable of stimulating normal human breast cell proliferation. The dose of the pure zeranol used to stimulate cathepsin D expression in MCF-7 cells (20 nM) is 18.97-fold greater. Therefore, the biological activity of meat extracts from zeranol-implanted beef cattle, in terms of estrogen-inducible or -dependent effects, may be more potent than that of zeranol in its pure chemical form.

3. *Zeranol suppresses PTP γ expression through estrogen receptor α , not estrogen receptor β :*

Evidence indicates that some xenoestrogens, particularly phytoestrogens, have a greater affinity for ER β than for ER α . Our recent studies have used the MCF-7 and MDA-MB-231 cell lines to help to elucidate which ER is involved in estrogenic suppression of PTP γ .

Results. MCF-7 and MDA-MB-231 cells were treated with 30 nM E2 or zeranol for 24 hours. Both agents suppressed PTP γ mRNA levels by ~56% in MCF-7 cells and these effects were completely blocked by 1 μ M of ICI 182,780. In contrast, E2, zeranol and ICI had no effect on PTP γ expression levels in MDA-MB-231 cells. Also, we found that untreated MCF-7 cells contained much higher mRNA levels of ER α than ER β (~200% higher), while MDA-MB-231 cells contained only ER β mRNA.

Significance. The findings indicate that ER α mediates zeranol- and E2-induced suppression of PTP γ mRNA levels in human breast cancer cells. This result is supported by our recent studies which, based on RT-PCR and immunohistochemistry methods, reveal an association between higher ER α expression and lower PTP γ expression in human breast cancer cells. Thus, an intact ER α pathway in human breast cancer cells may provide a means by which estrogenic agents, such as zeranol, can suppress PTP γ expression which may in turn lead to stimulation of cancer cell growth.

B. Evidence from human breast tissue culture studies:

A human breast tissue culture system was developed to study the effects of zeranol and other estrogenic agents on human breast under conditions that are more physiologically relevant than single cell type culture systems. Briefly, fresh human breast tissue specimens were divided into approximately 10 mg pieces and cultured on collagen sponges. Treatments were administered and tissues processed for molecular analysis and immunohistochemistry. These tissue culture studies have focused primarily on 2 areas of our interest: the effect of zeranol on PTP γ expression and the involvement of ER subtypes in zeranol-induced suppression of PTP γ .

Results. Normal human breast tissues (from reduction mastectomy patients) were treated with E2 or zeranol at various concentrations (0 – 50 nM) for 24 hours. RT-PCR revealed a dose-dependent decrease in PTP γ mRNA levels. Both E2 and zeranol induced nearly identical reductions in PTP γ mRNA expression at 20, 30 and 50 nM (~40%, ~75% and ~85% reductions, respectively). Furthermore, immunohistochemistry of cultured human breast tissues showed PTP γ staining to be localized to the epithelium. After 24 hours treatment with zeranol or E2 (30 nM), immunopositive staining was still present in the epithelium but was noticeably diminished.

RT-PCR and immunohistochemistry revealed that ER α expression is higher in human breast cancer tissues than in normal human breast tissues, while ER β expression is higher in normal human breast tissues than in cancerous human breast.

Significance. First, the results show that zeranol and E2 possess equivalent potencies with respect to suppression of PTP γ , suggesting that exposure to zeranol could elevate the total estrogenic burden and subsequently alter the expression of a putative cancer suppressor gene. Also, as mentioned previously, the degree of PTP γ suppression is much greater in the cultured breast tissues than in isolated human breast epithelial cells. Thus, the current tissue culture findings, in combination with the co-culture experiments described above, establish the importance of epithelial-stromal interaction in the estrogenic suppression of PTP γ . New avenues of research aimed at elucidating the details of this interaction can now be developed.

The differential expression of the ER subtypes in normal and cancerous breast tissues supports the cell culture studies, which suggest that zeranol- and E2-induced suppression of PTP γ is mediated by ER α . In light of our published results which revealed lower PTP γ expression levels in human breast cancer tissues than in normal breast tissues (Zheng et al., 2000), the current results indicate that lower PTP γ expression is associated with higher ER α expression.

C. Evidence from animal studies:

The intact female ACI rat is an estrogen-sensitive animal model for the induction of mammary tumors. Treatment of female ACI rats with the natural estrogen, E2, results in the formation of mammary tumors at high incidence. We felt that this animal model would provide a good system to study the effects of zeranol on mammary tumorigenesis and PTP γ expression. Ovary-intact, 8 – 9 week old ACI rats were treated with zeranol via sustained-release pellets (5 μ g/day for 112 days). Whole mammary tissues were collected for immunohistochemistry and RT-PCR to determine PTP γ mRNA expression levels.

Results. Under this treatment regimen, zeranol did not induce mammary tumors in the rats. However, PTP γ mRNA levels within the mammary gland were reduced by approximately 50% in the zeranol-treated rats. In addition, immunohistochemistry revealed that PTP γ was localized to the mammary epithelium.

Significance. The findings of this animal study are consistent with the in vitro results in human breast cells and cultured breast tissues described above in which exposure to the nonsteroidal estrogenic agent, zeranol, suppresses PTP γ mRNA levels. In addition, in both human breast tissues and in this ACI rat model, PTP γ was immunolocalized to the glandular epithelium. These results suggest that the ACI rat may be a good model for the continued study of the role of PTP γ in human breast. Furthermore, the zeranol-induced reduction in PTP γ mRNA expression levels, in the absence of observable histological changes in the mammary glands in comparison to control rats, suggests the potential value of the ACI rat model for the sensitive in vivo detection of estrogenic effects of putative xenoestrogens/endocrine disruptors. In addition, the results indicate that the dose or the duration of zeranol treatment needs to be elevated or lengthened to confirm the ability of zeranol to induce histological and perhaps neoplastic changes in the mammary gland of ACI rats. This contention is consistent with our hypothesis that the life-long exposure to low doses of zeranol (via consumption of zeranol-containing animal-derived food products) may pose a human health risk with respect to estrogen-sensitive tissues such as the breast. Thus, an appropriate animal model for the study of zeranol exposure and mammary tumorigenesis would likely involve extremely long treatment durations. Nevertheless, given the proposed role of PTP γ in cancers, including breast cancer, the ability of zeranol to induce changes in mammary PTP γ expression in an in vivo model strengthens the need for further investigation of the risk of exposure to dietary zeranol for human breast cancer.

Further research needed to address the potential risk of dietary zeranol for human breast cancer.

In order to confirm a role for dietary zeranol exposure in breast cancer etiology, associations must be established among (a) dietary intake of zeranol-containing meat, (b) zeranol levels in breast tissues, (c) changes in mechanism-based molecular markers of estrogenic exposure, and (d) breast cancer incidence. In addition, the levels of zeranol present in commercially available meat products must be determined. To this end, it would be necessary to develop new tools for the development of a new, rapid assay (such as an ELISA) for the measurement of zeranol in biological specimens and meat products. Such research would seek to link beef production practices, beef consumption and human breast cancer in an integrated and comprehensive approach that will clarify the potential role of zeranol exposure in breast cancer. Ultimately, information derived from our research should prove useful to federal regulatory agencies in making informed decisions regarding the status of current regulations and practices in the use of growth promoters in beef destined for human consumption.

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